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Cellular susceptibility and oxidative stress response to menadione of logarithmic, quiescent, and nonquiescent *Saccharomyces cerevisiae* cell populations

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Abstract

The aim of the present study was to compare cellular susceptibility and oxidative stress response of *S. cerevisiae* logarithmic (log), quiescent (Q), and non-quiescent (NQ) cell populations to menadione – a well-known inducer of oxidative stress. Three main approaches were used: microbiological – cell survival, molecular – constant field gel electrophoresis for detection of DNA double-strand breaks (DSB), and biochemical – measurement of reactive oxygen species (ROS) levels, oxidized proteins, lipid peroxidation, glutathione, superoxide dismutase (SOD) and catalase on *S. cerevisiae* haploid strain BY4741. The doses causing 20% (LD_{20}) and 50% (LD_{50}) lethality were calculated. The effect of menadione as a well-known oxidative stress inducer is compared in the log, Q, and NQ cells. Survival data reveal that Q cells are the most susceptible to menadione with LD_{50} corresponding to 9 μM menadione. On the other hand, dose-dependent DSB induction is found only in Q cells confirming the results shown above. No effect on DSBs levels is observed in log and NQ cells. Further, the oxidative stress response of the cell populations is clarified. Results show significantly higher levels of SOD and ROS in Q cells than in log cells after the treatment with 100 μM menadione. On the other side, higher induction of oxidized proteins, malondialdehyde, and glutathione is observed after menadione treatment of log cells. Our study provides evidence that *Saccharomyces cerevisiae* quiescent cells are the most

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susceptible to the menadione action. It might be suggested that the DNA damaging and genotoxic action of menadione in *Saccharomyces cerevisiae* quiescent cells could be related to ROS production.

Keywords
Menadione, quiescence, *Saccharomyces cerevisiae*, stress response

Introduction

Organisms have developed strategies to trigger a stress response when exposed to environmental challenges in order to restore cellular homeostasis (Tagkopoulos et al. 2008; Mitchell et al. 2009). The cellular stress response is thought to be universal and encompasses a range of cellular functions, including cell cycle control, repair of damaged proteins, stabilization and repair of DNA and chromatin, cell membrane repair, and more (Kültz 2005). In nature, cells may exist in a proliferative or non-proliferative state (Gangloff and Arcangioli 2017; Sun and Gresham 2021). The non-proliferative state includes quiescent or non-quiescent cells (Sun and Gresham 2021). As most of the cells in human tissues are non-dividing, quiescence is a major form of life (Gangloff and Arcangioli 2017).

Based on this understanding cellular quiescence is of great importance, especially since studies performed on quiescent cells are still scarce. Such studies in multicellular organisms are difficult because of the complexity of the signals that control them. One of the possible solutions is the application of quiescent yeast cells as it is believed that they function similarly to the mammalian and human cells and share similar mechanisms and the same set of genes involved in the quiescence (Gangloff and Arcangioli 2017; Daskalova et al. 2021a).

Saccharomyces cerevisiae is a widely used test system for studying oxidative stress and its related consequences. Results obtained on *S. cerevisiae* could be easily extrapolated at mammalian, including human level because of homology in genes and conservative functions of proteins (Foury 1997; Hartwell 2004; Wright et al. 2014). Thus, the application of quiescent cells may provide a suitable platform for studying the effect of various toxic compounds on mammalian and human cells.

The aim of the present study is to compare cellular susceptibility and oxidative stress response to menadione of *S. cerevisiae* logarithmic (log), quiescent (Q), and non-quiescent (NQ) cell populations.

Materials and methods

Saccharomyces cerevisiae strain BY4741

Saccharomyces cerevisiae BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) obtained from the EUROSCARF collection was used in the present work. The growth curve of *Saccharomyces cerevisiae* BY4741 on YEPD medium is provided as a Suppl. material 1: Fig. S1. Yeast cells were grown on a standard yeast extract-peptone-dextrose (YEPD) medium

at 30 °C, 204 rpm for 168 h. Yeast media were prepared as described by Sherman et al. (2001). The growth curve of the strain is provided as a Suppl. material 1: Fig. S1.

Samples were withdrawn at exponential (24 h) and late stationary phase (168 h). Quiescent (G_0) and non-quiescent cells were isolated from stationary phase yeast population (168 h) according to the protocol described by Allen et al. (2006). In brief, yeast biomass in stationary phase (OD540 = 200 (2×109 cells/ml) was layered on Percoll density gradient and after centrifugation at 400 g (60 min at 20 °C) two layers of cell fractions were formed – the denser one composed of Go (Q) cells (lower fraction) and a less dense fraction of NQ cells (upper fraction). Both fractions were separated and microscopically examined. Go cells were characteristically rounded with thickened cell walls, and no budding cells were observed – these morphological features are typical for the cells in Go state. For comparison, the stationary phase cell population of NQ cells (upper fraction) was heterogeneous – both budding, elliptical cells, and deformed, granular and non-budding cells were observed.

Cell survival

Cell suspensions with concentration 1×10^7 cells/ml were treated with various concentrations of menadione (2-methyl-l,4-naphthoquinone, synthetic form of vitamin K) in the range 1–200 μM for 60 min at 30 °C, 200 rpm. Cells were then centrifuged (825 g), the supernatant was removed and the pellet was resuspended in a liquid YEPD medium. Cells were plated on a solid YEPD medium and incubated at 30 °C for 3 days to evaluate the survival. Doses of lethality (LD_{20}, and LD_{50}) were calculated (Lidanski 1988) by the following formulae:

$$lgLD50=lgA+(lgB-lgA)/((50-A)/(B-A))$$
$$lgLD20=lgA+(lgB-lgA)/((20-A)/(B-A)),$$

where A – the closest smaller than 50 or 20%, respectively, lethality percentage; lgA- lg of the concentration corresponding to A; B – the closest higher than 50 or 20%, respectively, lethality percentage; lgB- lg of the concentration corresponding to B.

Cell-free extracts

Isolation of cell-free extracts from log, Q, and NQ cells was carried out according to the procedure described by Daskalova et al. (2021b) and were used for further biochemical analyses.

Constant field gel electrophoresis (CFGE)

CFGE for detection of DNA double-strand breaks (DSBs) was applied as described in Todorova et al. (2015, 2019). The levels of DSB induced presented as a mean fraction of DNA released (FDR) from the wells was quantified by measurement of ethidium bromide fluorescence using Gene Tool Analyser G: Box (Syngene) and calculated as described in Chankova et al. (2009).

Biochemical analysis

Oxidative stress markers assay

The redox state of logarithmic, quiescent, and non-quiescent yeast cells was assessed through measurement of intracellular levels of accumulated ROS (Kostova et al. 2008), levels of carbonylated proteins (Mesquita et al. 2014), and oxidized lipids (Hodges et al. 1999).

Glutathione measurement

The measurement of intracellular glutathione was carried out according to the procedure of Zhang (2000).

Enzymatic analysis

Superoxide dismutase (SOD) and catalase (CAT) enzyme activities were determined spectrophotometrically according to Beauchamp and Fridovich (1971) and Aebi (1984), respectively.

Protein content

Total intracellular protein was determined according to Lowry et al. (1951). As a standard, bovine serum albumin (Sigma St. Louis, MO, USA) was used.

Data analysis

The experiments were repeated at least three times from independently grown cultures. Data points in all the figures are mean values. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or less than the symbols. All the calculations were done with GraphPad Prism program, version 6.04 (San Diego, USA). The statistical analysis included the application of Student's *t-test* and One-way ANOVA followed by Bonferroni's *post hoc* test. $P<0.05$ was accepted as the lowest level of statistical significance.

Results

Resistance to menadione measured as cell survival

Our first step was to determine the cell survival of the three cell populations after treatment with 100 μM menadione. Data revealed that the log cells are the most resistant to menadione action (Fig. 1A). Further experiments with log and Q cells were performed in order to determine the potential dose-response (Fig. 1B). A dose-dependent decrease in cell survival was obtained for both populations, better expressed in the Q cells.

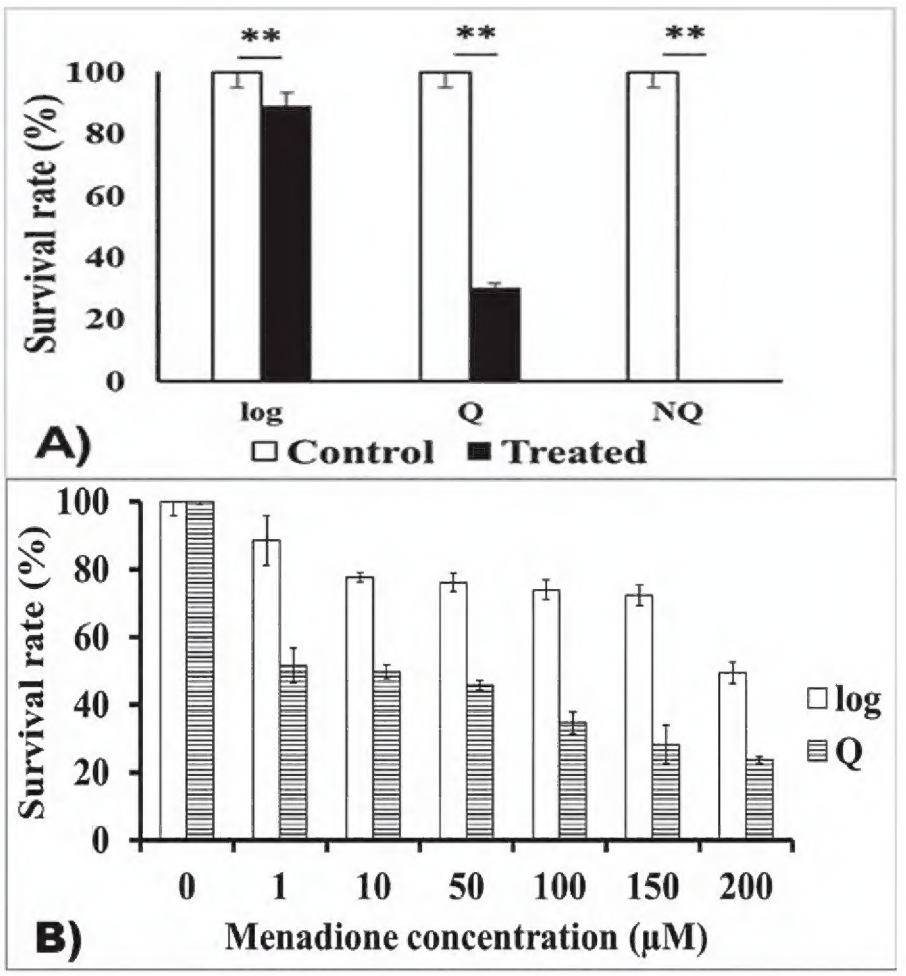

Figure 1. Cell survival after menadione treatment **A** effect of 100 µM menadione on log, Q, and NQ cell populations **B** effect of menadione in a concentrations' range of 1–200 µM on log and Q cells. Each value represents the mean ± SEM (Standard error of the mean) (n = 3).

Table 1. Levels of lethality calculated after menadione treatment.

Cell populations	LD_{20} (µM)	LD_{50} (µM)
Log	35	199
Quiescent	0.65	9

Two levels of lethality were calculated: LD_{20} and LD_{50} (Table 1).

Further, the levels of DSB induced were compared. Our results confirmed the ones obtained for cell survival. Dose-dependent DSB induction is measured only in quiescent cells (Fig. 2). The DSB levels measured after the treatment with 150 µM menadione were 1.5-fold higher than the spontaneous ones. No effect on DSBs levels is observed in log and NQ cells.

Further experiments were focused on studying the potential differences in the susceptibility based on various markers for oxidative stress – reactive oxygen species, oxidized proteins, malondialdehyde, intracellular glutathione, superoxide dismutase, and catalase.

Concentration of reactive oxygen species (ROS) after menadione treatment

The ROS measured in the three cell populations are presented in Fig. 3A. The levels measured in G_0 cells after menadione treatment are significantly higher – around 3-fold than those measured in the controls. There is a statistically significant but biologically insignificant effect on the ROS levels in log cells. This observation is in a good

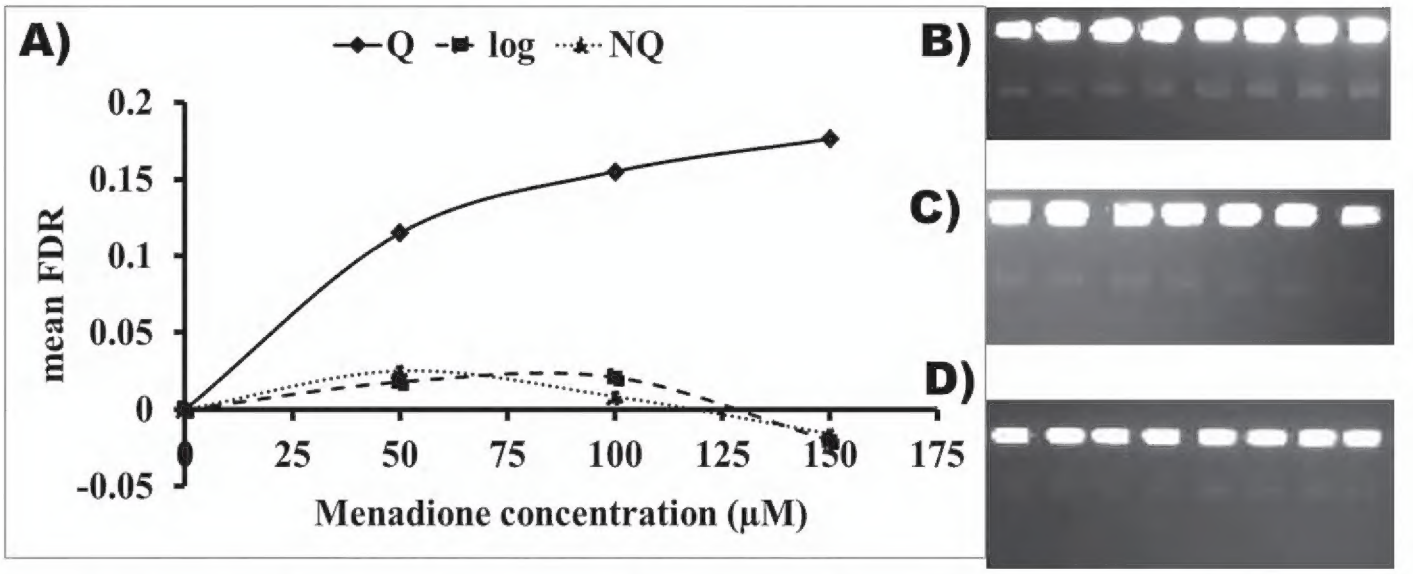

Figure 2. DSBs induced by various concentrations (50–150 μM) of menadione **A** induction of DSB presented as normalized FDR **B** Q cells **C** log cells **D** NQ cells.

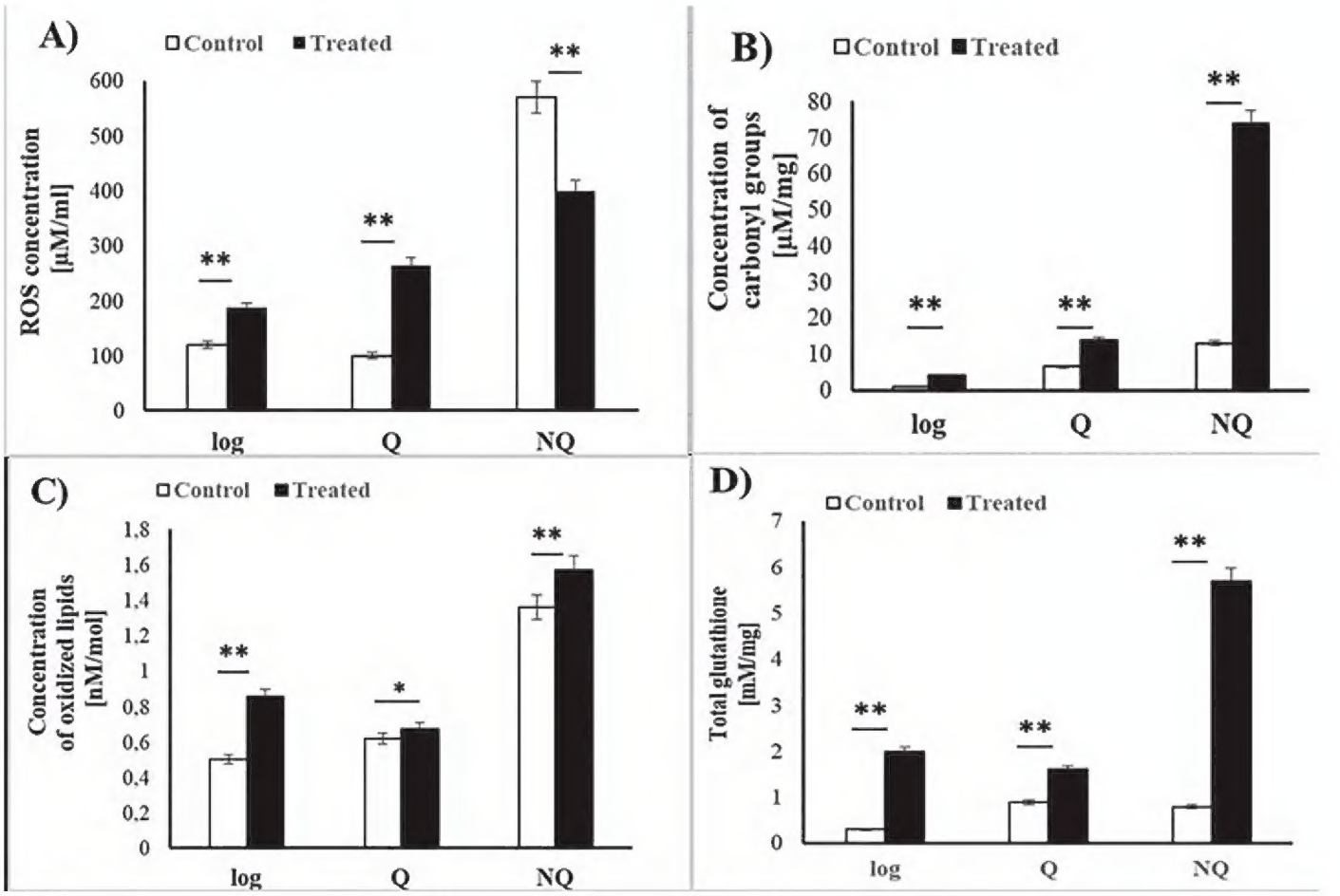

Figure 3. Comparative analysis of the levels of reactive oxygen species **A** oxidized proteins **B** malondialdehyde **C** and total glutathione **D** in *S. cerevisiae* logarithmic (log), quiescent (Q), and non-quiescent (NQ) cell populations after the treatment with menadione. Each value represents the mean ± SEM (Standard error of the mean) (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

correlation with the cell survival and the DSBs induced in Q cells in comparison with those observed in log cells.

The constitutive levels of ROS, oxidized proteins, and MDA in NQ cells were significantly higher than those measured in log and Q cells. Treatment with 100 μM menadione resulted in significant induction of oxidized proteins and glutathione (Fig. 3B, D). Interestingly, the ROS levels measured in NQ cells were lower after the menadione treatment in comparison with the control levels (Fig. 3A).

Concentration of protein carbonyl groups

Data presented in fig. 3B provides information concerning the concentration of protein carbonyl groups. Comparing the constitutive levels, around 7-fold higher levels were measured in Q cells in comparison with the log ones. This could be explained as a result of the cells' starvation. Although, the highest quantity – 14 μM/mg was determined in Q cells the induction was only around 2-fold. Higher induction – around 6-fold was measured in the log cells.

Levels of malondialdehyde (MDA)

Concerning the MDA, comparatively equal constitutive levels were observed between Q and log cells (Fig. 3C). The NQ cells showed significantly higher MDA levels. As a result of the menadione treatment the most significant induction of MDA (around 2-fold) was measured in log cells ($p < 0.001$).

Concentration of glutathione

The GSH concentration in untreated Q cells was 3-fold higher than that in log cells. Interestingly, menadione treatment did not result in a significant induction of GSH compared to the untreated control. The GSH concentration was only 2-fold higher (Fig. 3D). At the same time, the treatment with 100 μM menadione resulted in 7-fold higher GSH levels in log cells.

Antioxidant enzyme (Superoxide dismutase and Catalase) activity after menadione treatment

Concerning the constitutive levels of the antioxidant enzymes superoxide dismutase and catalase, differences were obtained. The catalase levels were comparable in the three cell populations, while SOD was lower in Q cells than in the log and NQ cells (Fig. 4A, B).

Significant induction of SOD was observed in Q cells after the application of menadione (Fig. 4A). No effect was obtained concerning the catalase levels (Fig. 4B).

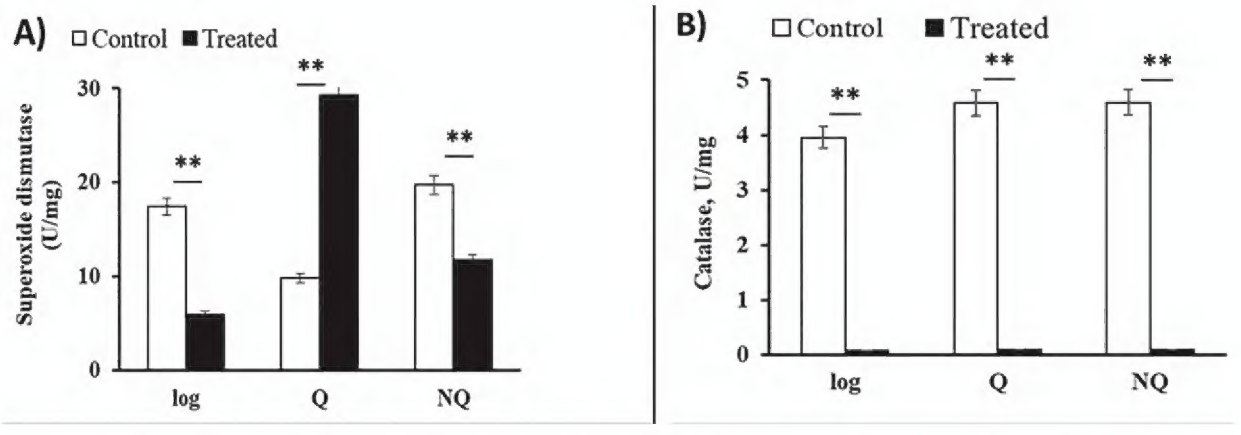

Figure 4. Comparative analysis of the response to menadione based on the enzymatic antioxidant system **A** superoxide dismutase and **B** catalase presented as units/mg. Each value represents the mean ± SEM (Standard error of the mean) (n=3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

Discussion

Data presented here provide a comparative analysis of the cellular susceptibility and oxidative stress response to menadione of logarithmic, quiescent, and nonquiescent *Saccharomyces cerevisiae* cell populations. Differences in the cellular susceptibility are obtained depending on the endpoint used. Based on cell survival, DSBs induction, ROS, and SOD Q cells are more susceptible to menadione. On the other side, higher induction of oxidized proteins, MDA, and glutathione is observed following menadione treatment of log cells.

The measured increased ROS levels in Q cells correspond well with the decrease in cell survival and the well-expressed DSB induction. The cytotoxic mechanism of action of Menadione in G0 cells is stronger, probably due to lower metabolic activity and higher oxygen levels in the cells. This is in accordance with the report by Fabrizio and Longo (2008) that quiescent cells are characterized with lower energy consumption and ADP content, which may lead to increased intracellular oxygen levels and single-electron oxygen reducers. Such conditions may occur during the chronological aging of yeast cells. On the other hand, the decrease in ROS levels measured after the treatment with menadione of NQ cells could be explained by the higher percentage of cells in a terminal state and entering apoptosis (Davidson et al. 2011).

It is already reported that the toxicity of quinones including menadione in *S. cerevisiae* depends on the oxygen presence (Rodrigues-Pousada et al. 2004). This could be explained by their possible role as catalyzers in the ROS generation via redox-cycling activity. The cellular response to menadione has been shown to be associated with the induced synthesis of a large number of proteins, some of which are specific and are synthesized only upon exposure to this toxic agent (Flattery-O'Brien et al. 1993).

In the present work, log cells showed increased levels of oxidized proteins, MDA, and glutathione. This could be explained by their increased metabolic activity and a higher rate of protein synthesis (Daskalova et al. 2021b). Stress-induced toxic oxygen species, such as superoxide and hydroxyl radicals, damage biological membranes and other cellular macromolecules, leading to mutations, cancer, or cell death. A direct indicator of the onset of these processes is the appearance of carbonyl groups in proteins, as well as lipid peroxidation. In addition, the formation of ROS is inevitable under aerobic conditions due to the reactive nature of molecular oxygen. The action of these factors individually or jointly can lead to the appearance of oxidative stress – acute or chronic (Petrova and Kujumdzieva 2010). Oxidative processes that take place during oxidative stress may lead to reversible or irreversible functional changes in proteins, which are the main reason for cellular dysfunction. Protein changes are associated with the formation of carbonyl groups in them. Biochemical analyses have shown that carbonyl groups introduced into the side chains of specific amino acids in the active site of enzymes trigger the initial steps in the degradation of these proteins (von Herrath and Holzer 1985; Levine and Munro 2002; Grimsrud et al. 2008; Apoorva et al. 2020).

Lipid oxidation occurs through the interaction of ROS with fatty acids in the membrane lipid layer. This changes the functionality and permeability of biological membranes and also leads to other disorders. Cell death can be caused by the release

of cell contents as a result of these changes. Malonaldehyde is the end product of lipid oxidation. It accumulates in cells and is a highly reactive and toxic electrophilic compound that can form covalently bound products with different proteins. Its concentration in the cell is used as a biomarker to account for the influence of stress agents. In our work, the MDA levels remained similar in control and treated Q cells. One of the explanations could be the thicker cell wall (Daskalova et al. 2021b).

Glutathione plays an important role in protecting the cell against oxidative stress by protecting it from the toxic effects of ROS through its involvement in mechanisms for detoxification and regeneration of important cellular antioxidants (Valko et al. 2006). The antioxidant function of this tripeptide is directly related to the reduction state of the oxidized GSSG / reduced GSH glutathione pair. More than a few dozen genes have been identified whose transcription is affected by redox balance in the cell (Allen and Tresini 2000). It has been found that the GSH: GSSG ratio is of major importance for this regulation. The glutathione system serves as a cellular redox buffer and changes in GSH: GSSG balance can lead to oxidation of redox-sensitive cysteine residues in various proteins (Rahman 2005). Therefore, the increase in intracellular glutathione content may be one of the adaptive mechanisms to stress in the yeast *S. cerevisiae*. Glutathione is a compound with antioxidant and antielectrophilic activity, which suggests its role in the resistance of cells in a medium with menadione. The accumulation of oxidized glutathione in the cell is an important parameter for measuring the level of oxidative stress.

All enzymes in glutathione metabolism work in an integrated way, allowing the cell to adapt to different stress conditions (Hayes and Pulford 1995), with de novo glutathione synthesis being the most important mechanism for increasing levels of reduced GSH in response to oxidative stress (Rahman 2005). However, the oxidized/ reduced glutathione pair (GSSG/GSH) ratio before and after treatment with menadione remained relatively constant in G0 cells. Controlled changes in GSSG / GSH contribute to the maintenance of cellular redox potential, which determines resistance to toxic effects. The stable GSSG / GSH ratio also indicates that in cells of *S. cerevisiae* BY4741 strain, menadione exhibits its toxicity through its redox-cyclic mechanism of action associated with the generation of reactive oxygen species rather than by interaction with reduced glutathione in the cell. In the second case, this would lead to the formation of menadione – S - glutathione conjugates, accompanied by a sharp decrease in the concentration of intracellular glutathione.

Our study provides evidence that *Saccharomyces cerevisiae* quiescent cells are the most susceptible to the menadione action. It might be suggested that the DNA damaging and genotoxic action of menadione in *Saccharomyces cerevisiae* quiescent cells could be related to ROS production.

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Supplementary material 1

Figure S1

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Data type: jpg file

Explanation note: Fig. S1. Growth curve of Saccharomyces cerevisiae BY4741 and glucose assimilation in batch cultivation on YEPD media at 30 °C, 204 rpm for 168 h.

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